STUDIES ON THE MECHANISM OF CERULOPLASMIN CATALYZED REACTIONS

BY

PER-OLOV GUNNARSSON

1974

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INTRODUCTION

The food eaten by animals and bacteria is only in part transformed into new cellular material; the rest is broken down, with the formation of CO_2 and H_2O to yield energy. This breakdown process is the sum of a long series of oxidation-reduction reactions and requires the presence of suitable electron acceptors for the electrons liberated in these reactions from the degradation of the foodstuff molecules. Molecular oxygen, which is used by most organisms as the terminal electron recipient, is a very inert gas under physiological conditions and is unsuitable as an electron acceptor in the organism without the intervention of some catalytic system. The enzymatic catalysis of this oxygen reduction is fundamental for aerobic life. An understanding of this reaction is thus of considerable importance, but the detailed chemistry and structures involved have nevertheless so far remained obscure.

In eucaryotic cells the final step in the conversion of oxygen to water is catalyzed by cytochrome c oxidase, the terminal enzyme of the mitochondrial electron transport chain. Direct reduction of oxygen to water is not a very common reaction in living cells, but a few other enzymes are known that can accomplish this: ceruloplasmin from mammalian blood plasma, laccase from various plant sources and ascorbate oxidase from higher plants. All these oxidases are metalloproteins; cytochrome c oxidase contains two heme-bound iron ions and two copper ions; the other enzymes contain at least four copper ions per unit. At present it seems that all enzymes catalyzing the conversion of oxygen to water require prosthetic copper for their activity.

The copper ions in these proteins do not have identical properties. Optical and electron paramagnetic resonance (EPR) spectra allow three different forms of

copper to be distinguished (1-5). Type 1 paramagnetic copper is characterized by an EPR spectrum showing a hyperfine splitting that is unusually narrow for a copper complex. This type of copper is associated with an extensive visible absorption band around 600 nm, and is thus responsible for the intense blue colour of some copper-containing proteins. The molar absorption coefficient of ceruloplasmin at 610 nm is 11300 (6), which is several orders of magnitude higher than in most simple Cu^{2+} complexes. A second form of EPR-detectable copper is present in several oxidases. This type 2 copper exhibits less remarkable optical and EPR properties than those of type 1 copper. The recognition of this component was complicated because it is very similar to copper in denaturated proteins or in simple complexes (4,7). The third form of copper gives no EPR signal and has for long been assumed to be a stable form of Cu^{+}. About half of the total copper in oxidases such as ceruloplasmin and laccases is of this "non-detectable" type. Redox titrations now suggest that these diamagnetic copper ions may exist in the oxidized protein as a spin-paired Cu^{2+} - Cu^{2+} couple (1,8,9).

Ceruloplasmin is present in serums to an extent of about 30 mg per 100 ml. 95% of the copper in serum is bound to ceruloplasmin, which with a copper content of 0.32 % and a molecular weight of 134000 appears to contain six or seven copper atoms per molecule (10,11). Of this copper about 45 % is EPR-detectable. Little is known about the roles of the different types of copper atoms in ceruloplasmin. However, it has been suggested that the blue, type 1 copper is involved in the initial interaction between enzyme and substrate, while the diamagnetic copper functions as an electron acceptor possibly acting in the reduction of oxygen (4,8,12).

Among numerous inhibitors of ceruloplasmin, there is a class of anions such as azide, cyanate, thiocyanate, cyanide and halide, which it has been suggested, may affect the enzyme activity by binding to copper (13-15). The binding of these anions has since been reported to cause changes in optical and EPR spectra. It has often been assumed that type 2 copper is involved in the anion binding by the protein (15-18). Type 2 copper in other copper-containing enzymes also shows a high affinity for certain anions (5,18,19), and fluoride is so strongly bound to fungal laccase, that preparations of this enzyme are usually contaminated with fluoride (18). Little is known concerning the function of the type 2 copper in oxidases, but their anion-binding capacity has given rise to the proposal that this form of copper stabilises some negatively charged intermediate in the reduction of oxygen, such as a peroxide anion (4).

There has been much controversy about the substrate specificity of ceruloplasmin. Curzon et al (20) stated that Fe^{2+} was a substrate for the enzyme and Curzon later showed that Fe^{2+} increased ceruloplasmin activity towards N,N-dimethyl-p-phenylenediamine (DPD) (21). A coupled oxidation mechanism was proposed in which Fe^{2+} is oxidized to Fe^{3+} by ceruloplasmin and then regenerated to Fe^{2+} by DPD. This electron cycling effect produced by contaminating iron can be eliminated by the use of chelators such as EDTA. McDermott et al (22) classified the reported substrates into three groups: 1. Ferrous ion, 2. Certain aryldiamines and polyphenols that are oxidized directly by the enzyme and 3. A variety of compounds that appear to be oxidized via a shuttle such as iron. Recently, however, there have been doubts about the validity of this model. Many of the compounds oxidized via an iron-shuttle seem to be true substrates for ceruloplasmin (23,24).

The mechanistic interpretation of kinetic data for ceruloplasmin catalyzed reaction has been complicated by the fact that non-linear Lineweaver-Burk graphs are obtained for the oxidation of DPD. This has led to the suggestion that either the enzyme has two kinds of active site (25,26), or that ceruloplasmin preparations contain two kinetically different species (27). More recent investigations by Pettersson (28,29) have established that the non-linear rate-behaviour is due to the primary product obtained (DPD^+) itself functioning as a substrate for ceruloplasmin. Furthermore DPD^+ undergoes a rapid non-enzymatic dismutation reaction during the oxidation process, which together with a non-enzymatic decomposition of the second reaction product, DPD^{2+} also contributes to the complexity of the kinetics of the reaction system. In the reports summarized in the present thesis, consideration has been paid to the elimination or correction of the effects of such processes.

The aim of the experiments described in this thesis has been to contribute to a clarification of the substrate specificity and the reaction mechanism of ceruloplasmin and especially of the nature of the interaction of inhibitors and reducing substrates with the enzyme. This topic is of considerable interest since the function of ceruloplasmin is still a matter of debate. Further, a detailed knowledge of the mechanism of ceruloplasmin is desirable, not only since it is in itself an interesting enzyme, but also because it may provide a key to the mechanisms of other copper-containing oxidases such as cytochrome c oxidase, which are important for the utilization of oxygen in aerobic organisms.

INHIBITION OF CERULOPLASMIN

Inhibition by carboxylic acids

Unsaturated and aromatic carboxylic acids have been reported to inhibit the oxidase activity of ceruloplasmin (13). This inhibition is reported not to be competitive with respect to the reducing substrate dimethyl-p-phenylenediamine (DPD), although many carboxylic acids are substrates of ceruloplasmin (24). This is true in the sense that no inhibitor has been found to give rise to the characteristic rate behaviour of competitive inhibition in a one-substrate-system. However, ceruloplasmin is known to operate by a two-substrate-mechanism, in which oxygen is the second substrate, and the enzyme oscillates between oxidized and reduced states during the catalytic cycle. Furthermore, earlier inhibition studies were carried out before the complexity of the ceruloplasmin-DPD system was recognized by Pettersson (28,29). Paper III describes a kinetic investigation of the inhibitory action of some carboxylic acids on the enzymatic oxidation of DPD. The calculations of reaction rates and substrate concentrations include corrections for dismutation equilibria and other nonenzymatic reactions affecting the kinetics of the reaction system.

Statistical analyses carried out for inhibition by fumaric, maleic and benzoic acid using a simplified model mechanism, gave no evidence for any difference in ligand binding to the oxidized and reduced forms of the enzyme. This indicates that enzyme-inhibitor complex formation is independent of the redox state of the enzyme (III). Further, no evidence was obtained for kinetically significant formation of a ternary enzyme-substrate-inhibitor complex with any of the carboxylic acid tested. This result might be due to competition between substrate and inhibitor for the

same binding site, but an equally likely possibility is that enzyme-substrate complexes are kinetically insignificant also in the absence of inhibitors. However, the lack of correlation of stability constants with structural similarities, or with the energy levels of the highest occupied molecular orbital in the inhibitors, appears to argue against a competitive inhibition mechanism.

Inhibition by inorganic anions

The interaction between ceruloplasmin and inorganic anions such as azide, cyanide, cyanate, thiocyanate and fluoride has been the subject of repeated and detailed study (13-15,30,31). It was early suggested that this class of inhibitor affects the oxidase activity of the enzyme by binding to protein copper. Changes in optical absorbance and electron paramagnetic resonance spectra of the protein in the presence of inhibitory anions led to the conclusion that these anions interact strongly with the type 2 copper of the enzyme (18).

Although there is an extensive literature, dealing with the action of inhibitory anions on ceruloplasmin, there is relatively little information available on kinetic inhibition constants. The reason for this seems to be that the steady-state rate behaviour of the enzyme has been poorly understood. A study of anion binding to ceruloplasmin based on the steady state kinetic interpretation made by Pettersson (28,29), and using the simplified model mechanism put forward in paper III, is described in paper IV. These experiments indicate that halide ions interact with both oxidized and reduced enzyme, although inhibition occurs mainly through complex formation involving the reduced form of the protein. Complex formation between azide or cyanate and the oxidized form of the enzyme, was found to be kinetically insignificant at low concentrations of the inhibitor, but the interaction

between reduced enzyme and anions was found to be very strong and it is this which is responsible for the great inhibitory effect on the ceruloplasmin oxidase activity.

The kinetic stability constant for the reduced enzyme-azide complex ($\geq$500 mM^{-1}) reported in paper IV, is in good agreement with previous data given by Curzon (13,31). However, these constants are much higher than those found in optical absorbance and EPR spectroscopic studies (15,32). It is therefore evident that spectroscopic measurements on the resting oxidized enzyme are not relevant to the cycling enzyme. Recently Beyers et al have interpreted their spectroscopic data in terms of two azide binding sites per molecule of ceruloplasmin in the oxidized state (33). Both sites were presumed to involve copper, but none of the complexes exhibit such a high stability constant as the complex between azide and reduced enzyme. They therefore suggested that the increased binding strength in the reduced enzyme must arise from the nature of the copper environment and not just from the nature of the copper.

Analysis of the kinetic data for anion inhibition, further established that formation of ternary enzyme-substrate-inhibitor complexes is kinetically insignificant (IV). By analogy with inhibition by aromatic carboxylic acids (III) this kinetic pattern is consistent with a competitive inhibition mechanism, but could also indicate that formation of enzyme-substrate complexes is kinetically insignificant also in the absence of inhibitors. The lack of common structural similarities between substrates, inhibitory carboxylic acids and inhibitory anions, and the kinetically established differences in the mechanism of action of the two groups of inhibitors, lend strong support to the latter idea.

EFFECT OF pH ON THE STEADY-STATE BEHAVIOUR OF CERULOPLASMIN

Pettersson used statistical methods to show that the steady-state rate equation at pH 4.8 is of the Michaelis-Menten type with respect to both DPD and DPD^+ (29). In paper I the significance of the rate equation established at pH 4.8 has been studied within the pH-range 4.5-6.0, and attempts have been made to characterize the effect of pH on the ceruloplasmin-catalyzed oxidation.

Interpretation of the pH-dependence of the oxidation of DPD was complicated by the fact that the diammonium ion $DPD \cdot 2H^+$ titrates as a dibasic acid in a conventional acid-base titration, while DPD^+ was found to have ampholytic properties. According to the criterion that all aromatic substrates for ceruloplasmin are characterized by highest occupied molecular orbital of extremely high energies, it seems improbable that the two most protonated forms would function as substrates for the enzyme between pH 4.5 and 6.0. The four remaining compounds were assumed to act as substrates and kinetic coefficients were estimated for these. Examination of this data gave no evidence of the presence in the rate equation of higher degree terms involving substrate concentration.

In an earlier investigation the apparent maximum activities (V) of the substrates DPD and DPD^+ seemed to be of the same magnitude (29). As can be seen from the pH-profiles of the V-values in paper I, this relationship holds for the whole pH-range examined. This observation suggests that the rate-limiting step in ceruloplasmin action is relatively independent of the nature of the substrate. It is of interest that later results obtained by Young *et al* have supported this assumption (24). The V-values varied over only an eightfold range for the 37 substrates tested by them, and values for the 20 para-amino compounds over only a two-fold range.

The pH-profiles of the maximum activities show a significant decreasing above pH 5.5. This might indicate that enzyme activity is dependent upon a group with a pK_a-value of about 6, for example an imidazole, which several investigators have reported to be of great importance in enzymatic activity or in copper binding (34-36).

SUBSTRATE SPECIFICITY OF CERULOPLASMIN

Relationship between K_m-values and molecular orbital characteristics of substrates

Unlike maximum activities, the K_m-values for the substrates tested in paper I, seem to be independent of pH. The K_m-values for different ceruloplasmin substrates also differed from V-values in showing marked variation. It was emphasized earlier that aromatic substrates for ceruloplasmin are characterized by extremely high energies of the highest occupied molecular orbital (EHOMO, which is known to be an appropriate measure of the electron-donor properties of a compound) (37). The K_m-values in paper I indicate a variation with electronic rather than steric properties, and in fact it is shown that K_m is positively correlated with the EHOMO-values. The approximately linear relationship obtained in plots of log K_m against EHOMO confirms that the correlation is exponential rather than linear. This probably reflects substrate-dependent differences in the activation energy for some initial reaction step. The lack of obvious steric restraints on substrates is confirmed by Young *et al* (24), although the authors suggested that negatively charged carbocyclic groups may play some repulsive role in the process of substrate-binding. The possible inhibitory action of the carboxylic acids was however not considered in the latter investigation.

Aniline derivatives as substrates for ceruloplasmin

Aromatic compounds with at least two electron-donating groups, such as aromatic diamines and catecholamines, are known to be substrates of ceruloplasmin (22). Curzon and Speyer have pointed out that related compounds with single electron-donating groups are neither substrates nor inhibitors of the enzyme, and suggested that at least two electron-donating groups are necessary for complex formation at substrate binding sites. However, EHOMO-values for some N-alkylated anilines, are sufficiently high to characterize these compounds as substrates of ceruloplasmin by the criterion given in paper I. The experiments described in paper II provide evidence, that N-alkylated aniline derivates are ceruloplasmin substrates. This result eliminates the hypothesis, that two electron-donating substituents of aromatic substrates are necessary for oxidation by ceruloplasmin. It also supports the earlier assumption that electronic rather than steric characteristics are of prime importance for the activity as a ceruloplasmin substrate.

Ascorbate as a substrate for ceruloplasmin

The substrate specificity of ceruloplasmin has been the subject of much controversy. In particular there have been conflicting reports about enzyme activity with ascorbate (38-40). In the model for substrate specificity proposed by McDermott (22), ascorbate was suggested to be a member of the class of substrates oxidized via a shuttle such as iron. In paper V, however, evidence is given that ceruloplasmin is reduced by ascorbate, as well as by some other compounds of the same group in the presence of 0.5 mM EDTA as chelator. The reduction is not significantly affected by addition of ferrous ion or by ten-fold variation of the EDTA-concentration, indicating that these compounds are true and direct substrates for ceruloplasmin. Similar results have recently been reported by other authors (23,41), and it seems most likely that compounds reducing the enzyme via iron may

also be able to reduce the enzyme directly, although usually at a considerably lower rate.

THE INITIAL REDUCTION OF CERULOPLASMIN

The paramagnetic form of protein copper (type 1 copper), which shows a major absorption band at 610 nm, is responsible for the blue colour of ceruloplasmin (15,42). Kinetic and EPR studies have suggested that this form of copper is reduced more rapidly than other electron accepting sites in the protein, and it is possible that electrons enter from the substrate via this form of copper (12,43). In a series of subsequent reactions, which include the rate-limiting step of the catalytic process, the protein is transformed into a form that undergoes rapid reoxidation with transfer of the redistributed electrons to oxygen.

Little is known, however, about the mechanism of the initial interaction of the substrate molecule with the enzyme. Paper V presents a stopped-flow kinetic investigation of the decolorization of the 610-nm chromophore by ascorbate. The reduction is due to a rate-determining second order interaction between enzyme and substrate. Further there is a linear correlation between reciprocal K_m-values and second-order rate constants determined from the reduction of the 610-nm chromophore by various substrates. It has earlier been emphasized that K_m-values are exponentially related to the energy of the highest occupied molecular orbital of the reducing substrate (I). In view of the present results, it may be concluded, that the orbital energy/K_m relationship reflects a corresponding relationship between orbital energy and the second order rate constants for the initial reduction of the enzyme by different substrates. It therefore appears that the rate of reduction of type 1 copper is mainly determined by the energy required for ionization of the reducing substrate.

Previous experiments carried out by Carrico et al, suggested that under non-steady-state conditions the 610-nm chromophore may be affected by the redox state of other protein chromophores (for example the 340-nm chromophore) (12). It is important to underline that the results presented in paper V show that the decolorization of the 610-nm chromophore is an apparent first order process and hence is governed by a time-constant and not by a time-variable, indicating that this decolorization step is independent of the redox state of other electron acceptors in the protein; at least the 340 nm chromophore undergoes a significant reduction concomitantly with the reduction of the 610-nm chromophore. The present transient kinetic data can therefore be compared with steady-state kinetic parameters obtained previously. (I-IV).

Maximum activity of the enzymatic oxidation is essentially independent of the nature of the substrate (I,24). This is consistent with a substrate independent rate-limiting step. The reoxidation process has been observed to be unaffected by variations in oxygen concentration except under limiting conditions (42). From a steady-state kinetic point of view, these facts justify consideration of the catalytic mechanism under aerobic conditions in terms of a second order reduction of the enzyme (E) by substrate (S) followed by a first-order reoxidation of the reduced form E´of the enzyme:

$$E + S \xrightarrow{k_1} E' + P$$

$$E' \xrightarrow{k_2} E$$

In this simplified mechanism k_1 is identical with the second-order rate constant for reduction of the 610-nm chromophore and k_2 is an apparent first-order constant corresponding to the rate-limiting reaction step. According to this mechanism estimates of second-order reduction rate constants should agree in magnitude with previous calculations of the quotient V/K_m. The data presented in paper V show that such is the case.

The kinetics of ceruloplasmin-catalyzed reactions have previously been interpreted in terms of a mechanism involving the formation of a kinetically significant enzyme-substrate complex (42). Molecular orbital calculations have suggested that the substrate properties of a compound can be described by the magnitude of the energy of the highest occupied molecular orbital (I), and thus that substrate binding may occur through the formation of a charge-transfer complex. However, the results presented in this thesis provide no evidence for the presence of a kinetically significant enzyme-substrate complex (I-V). Furthermore direct experimental evidence for the formation of charge-transfer complexes during catalysis is lacking. Although these data do not exclude that charge-transfer complex formation may be of importance in the reaction between ceruloplasmin and reducing substrates, it seems more reasonable to consider the initial reaction between ceruloplasmin and substrate in view of a direct reaction between two redox couples, rather than in view of a classical enzyme mechanism involving the formation of a kinetically significant enzyme-substrate complex.

The established pH-dependence of the second-order decolorization of the 610-nm chromophore (V) shows a decrease when the pH is raised. The previously determined variation of V with pH (I), agrees closely in magnitude with the variation of the 610-nm rate constant with pH, and thus provides additional support for the simplified reaction mechanism postulated earlier in this section.

EFFECT OF pH ON ELECTRON-PARAMAGNETIC-RESONANCE SPECTRA OF CERULOPLASMIN

The observed pH-dependence of the rate of reduction of ceruloplasmin by ascorbate (V), is very similar to the pH-profiles obtained in ^{35}Cl nuclear magnetic resonance investigations of chloride ion interaction with ceruloplasmin

(44). As the enzyme exhibits EPR spectral changes over the range pH 5-7 (36), it seemed to be of considerable interest to see if there was any correlation between these findings.

EPR spectra of human ceruloplasmin recorded at different pH-values show that the hyperfine peaks assigned to type 1 copper are shifted upwards on raising pH from 4.5 to 7.0 (VI). Corresponding EPR experiments for porcine ceruloplasmin, however, show spectra that are essentially independent of pH changes. It may thus be concluded that the type 1 copper of human and porcine ceruloplasmins behaves differently when pH is altered, although as has been previously pointed out, physicochemical properties of the two species are closely similar (11). As the rate constants for the reduction of porcine enzyme by ascorbate are of the same magnitude and display the same pH-profile as those obtained with human ceruloplasmin, it may be concluded that there is no obvious relationship between the pH-dependent variation in the kinetic behaviour and the EPR spectral properties of the enzyme.

Paper VI also includes a calculation of the relative intensity of the type 2 copper signal in EPR spectra of human and porcine ceruloplasmin. The type 2 copper accounts for 33-36 % of the total amount of paramagnetic copper in the enzyme. In view of recent evaluations of the molecular weight made by Ryden (11), it seems likely that only three of a total of 6 or possibly 7 copper atoms per molecule, are paramagnetic. Similar results on the proportion of the two different types of paramagnetic copper have been obtained by other authors (45,46). It thus appears reasonable to interpret the EPR spectra in terms of two different forms of type 1 copper, which respond differently to pH-changes. Simulated spectra based upon the assumption that ceruloplasmin contains one type 2 copper and two non-identical type 1 copper atoms, accord well with the experimental spectra.

CONCLUDING REMARKS

Since the isolation of ceruloplasmin was reported by Holmberg and Laurell in 1948 (47), there have been a number of investigations aimed at clarifying the reaction mechanism of this and other copper-containing enzymes. Detailed kinetic studies described in the present thesis have contributed to an understanding of the primary step of electron transfer from the reducing substrate to the enzyme. The results summarized here appear to eliminate some earlier hypotheses on the catalytic action of the enzyme. While there is some information available on the transfer of electrons from the enzyme to oxygen, the mechanism of redistribution of electrons within the enzyme still remains obscure and a clear answer to this question is probably closely associated with the problem of the structure and function of the different forms of copper in the enzyme and with the binding of the copper within the protein molecule. Much work undoubtedly remains to be done before a detailed knowledge of the reaction mechanism of copper-containing oxidases such as ceruloplasmin is at hand.

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